Factors affecting the efficiency of aerosolized salbutamol delivery via a metered dose inhaler and equine spacer device

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Short running title: Efficiency of aerosolised salbutamol delivery

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14 **Abstract**

15 Despite frequent use of metered dose inhalers (MDIs) and spacers in equine practice, limited
16 information exists on the efficiency of aerosol delivery using such devices. We determined the
17 particle size distribution within an MDI-generated salbutamol aerosol delivered via an equine
18 spacer using "best practice" delivery technique and assessed the effect of variations in MDI
19 use technique (shaking prior to each actuation, rapid repetitive actuations and MDI angulation)
20 on aerosol delivery efficiency.

21 Under optimal conditions, only 53(±18) microgrammes (μg) salbutamol per 100μg actuation
22 was delivered beyond the spacer. Although this aerosol had a high (89.6% [±2.4]) fine particle
23 (<5 micron [μm]) fraction, and a low mass median aerodynamic diameter (2.52 [±0.29]μm)
24 and particle size variability (geometric SD - 1.66 [±0.16]μm), within all particle size fractions
25 there was a high coefficient of variance (31-79%) of the percentage salbutamol delivered
26 between experimental runs, thus impeding any effort to predict drug delivery to the patient
27 during equine inhalation therapy. Despite observable trends and with the exception of minor
28 statistically significant changes in the least abundant particle sizes, none of the deviations from
29 a "best practice" delivery technique significantly altered the relative salbutamol delivery
30 beyond the spacer, a finding which has potential relevance with regard to maintaining user
31 compliance.

32

33 **Keywords:** horse, MDI, inhalation, aerosol, nebuliser

Introduction

The use of inhalation therapy in equine practice has recently increased in popularity, particularly in relation to corticosteroid and bronchodilator treatment of equine asthma (Robinson et al., 1993; Tesarowski et al., 1994; Derksen et al., 1996; Derksen et al., 1999; Durham, 2001) but also for the delivery of other therapeutic agents including antibiotics (Art et al., 2010; Burton et al., 2013; Ferrucci et al., 2013; Fultz et al., 2015). The proposed advantages over systemic drug delivery include a relatively lower cost, drug delivery directly to the site of action and, particularly in the case of corticosteroids, a reduced risk of systemic adverse effects (Hoffman, 1997, Duvivier et al., 1997; Duvivier et al., 1999; Lavoie, 2001). Various means of aerosol generation exist, including ultrasonic, jet and mesh nebulisation and metered dose inhalers (MDIs), each differing with respect to the variability in aerosol particle size distribution (Duvivier et al., 1997; Votion et al., 1997; Duvivier et al., 1999). Furthermore, a variety of delivery devices are available, including equine-specific and customised spacers and airtight facemasks, the use of which is indicated largely due to the inability to accurately synchronise aerosol generation with inspiration in the horse (Lavoie, 2001).

Although successful drug delivery to the peripheral airways is partly dependent the patient's breathing pattern and the viscosity, density, surface tension and hygroscopic growth potential of the drug solution (Silverman, 1990; Morrow, 1996), ultimately the aerodynamic diameter of the aerosolised particles is the major determinant of peripheral airway deposition (Stahlhofen, 1980). Despite the small size and low variability of the aerosolised particles generated by MDIs (Kim et al., 1985), there are a variety of factors which can significantly influence MDI-generated aerosol delivery to peripheral airways. In human respiratory medicine, this has led to established protocols for MDI use (Everard et al., 1995); protocols which have subsequently been applied to the field of equine inhalation therapy. However, despite many of the recommendations deriving from *in vitro* studies, there remains a lack of

concordance between the standard protocols used in human respiratory medicine and those employed in the laboratory setting (Everard et al., 1995). Such inconsistency has the potential to result in unnecessary recommendations being made to MDI users which may have a negative impact on patient compliance with appropriate self-medication. Such negative impact is likely to be amplified when unnecessary recommendations result in an extended duration of treatment, a significant consideration with equine inhalation therapy when multiple actuations are generally required. Recommendations which may significantly extend the duration of treatment include shaking the MDI prior to each actuation when multiple actuations are required and the avoidance of rapidly performed consecutive actuations (Everard et al., 1995; Wildhaber, 1996).

The limited data relating to drug delivery via an equine-specific spacer are largely derived from *in vivo* scintigraphic studies which revealed relatively poor and markedly varied aerosol delivery to the peripheral airways (Votion et al., 1997; Rush et al., 1999; Votion et al., 1999). Due to the lack of published *in vitro* studies on MDI-generated aerosol characteristics using equine spacers, this study was designed to measure the efficiency of delivery and particle size distribution of an MDI-generated salbutamol aerosol delivered via an equine spacer device. Specific deviations, regarded as having potential influence on owner compliance with respect to MDI use, from a "best practice" protocol, were evaluated in relation to their effect on the efficiency of aerosolised drug delivery.

Materials and methods

Three sets of comparative experiments were conducted within the study, each with a measured output of aerosolised salbutamol delivery to the various stages of a next generation impactor (NGI)[a], as follows: *Experiment 1* - Effect of shaking the MDI prior to each sequential actuation;

Experiment 2 - Effect of angulation of the MDI device within the spacer; *Experiment 3*; Effect of multiple actuations in rapid succession. Additionally, in light of the variability in data derived from experiment 1, selected data from experiments 2 and 3 were also used to measure the efficiency of salbutamol delivery with the MDI device secured in an optimal position relative to the spacer device (*Optimal delivery measurement*), whereby salbutamol retention within the spacer was also measured.

Salbutamol aerosol generation and delivery to the NGI

For comparative purposes, the quantity of aerosolised salbutamol delivered was determined by the number of 100µg actuations of the MDI[b] directly into an equine spacer[c] (Figure 1a). The spacer was connected to the throat of the NGI, a high-performance, precision, particle classifying cascade impactor designed for testing MDIs, dry powder inhalers, nebulizers and nasal sprays, separating aerosolised particles based on particle size and aerodynamic properties. The NGI is comprised of a throat (designed to mimic the calibre and airflow directional changes within the human upper airway) and a series of eight stages, characterised by different pore sizes of sequentially decreasing diameter, thus simulating the sequential decrease in airway diameter from the trachea to the terminal bronchioles (Figure 1b). Consequently, aerosolised particles delivered into the NGI are fractionated and collected onto each of these stages. The distal portal of the NGI was connected to a vacuum pump[d], calibrated to generate a constant flow rate of 60L/min through the entire system (spacer, throat, NGI and all connecting tubing). Leaks within the system were prevented by sealing all connections with parafilm[e] and the absence of leaks was confirmed by comparing airflow rate before and after each experimental run. Airflow was maintained for 30s after each experimental run. With a constant airflow of 60L/min, the stage effective cut off particle diameters (at 50% efficiency) were as follows:

stage 1 - 8.06μm; stage 2 – 4.46μm; stage 3 – 2.82μm; stage 4 – 1.66μm; stage 5 – 0.94μm; stage 6 – 0.55μm; stage 7 – 0.34μm; stage 8 - 0μm.

Following aerosol delivery, samples were retrieved from the spacer (*Experiments 2 and 3* only), throat and each collection stage by instilling 10ml distilled water, re-suspending any deposited salbutamol with a cell scraper, pipetting into a labelled container and storing at 4°C until further analysis. The decision to measure salbutamol deposition within the spacer was made following completion of experiment 1 which revealed a relatively low drug delivery to the NGI. This additional sample collection was conducted to determine whether, and to what extent, this low output reflected retention within the spacer. A separate pipette was used for each sample to reduce the risk of cross contamination. Following sample collection, the spacer, NGI stages and throat were washed in dilute detergent and rinsed with distilled water before being air dried, to avoid accumulation of static electricity. Prior to each experiment, the NGI, NGI stages, and throat were refrigerated at 4°C for 1h to minimise subsequent evaporative losses. Between experiments, the MDI was stored up-right at room temperature.

Salbutamol assay

Standard salbutamol concentrations (0 to 100μg/ml) were prepared from the stock solution (10mg/ml salbutamol hemisulphate salt[e] in distilled water) and 100μl of standard and sample (spacer, throat and NGI stages) was pipetted in duplicate into wells of a UV-clear flat bottom microwell plate[f]. Absorbance was read at 224nm and standard and sample concentrations calculated using multi-detection microplate data collection and analysis software[g]. A mean value of duplicate results showing acceptable agreement was used for subsequent statistical analyses.

Experimental designs

Within each set of comparative experiments, the order of runs was randomised. For each experiment, the MDI device was shaken for 30s prior to each run and two 'waste' actuations were performed prior to connecting the MDI to the spacer. Constant airflow was established prior to aerosol generation. All comparative experiments involved 8 repetitions of the delivery of 10 x 100µg actuations (total 1mg salbutamol) with the exception of *Experiment 3* (effect of rapid actuations), whereby 8 repetitions of 8 x 100µg actuations (total 0.8mg salbutamol) were delivered .The experimental designs are summarised in Table 1. Briefly, *Experiment 1* compared 8 repeats of 10 actuations delivered at 5 s intervals without removing the MDI from the spacer with 8 repeats of 10 actuations, each preceded by a 30s period of MDI shaking; *Experiment 2* compared 3 sets of 8 repeats of 10 actuations delivered at 5s intervals; each set differing with respect to the direction of actuation within the spacer (with the output nozzle horizontal or at 10° or 20° above the horizontal) (Figure 1c); *Experiment 3* compared 3 sets of 8 repeats of 8 actuations (MDI actuated in a horizontal direction), either delivered individually at 5s intervals, as 4 x double actuations in rapid succession (approximately 2 actuations per second) or as 2 x quadruple actuations in rapid succession (approximately 2 actuations per second); the *Optimal Delivery Experiment* measured the efficiency of salbutamol delivery under presumed optimal delivery conditions using selected data derived from *Experiments* 2 and 3 (horizontal actuation of the MDI and 5s interval between individual actuations).

Statistical analyses

For the comparative delivery experiments, values are presented as, and analyses applied to, measured salbutamol expressed as a percentage of the anticipated total aerosolised salbutamol actuated (median and range) (*Experiments 1 and 2* – 1mg; *Experiment 3* – 800µg). When only 2 experimental conditions were compared, a Mann Whitney test for non-parametric data was applied directly. When more than 2 experimental conditions were compared, a Mann Whitney test for non-parametric data was applied only if differences were revealed by a Kruskal-Wallis analysis. Significance was assumed at $P<0.05$. For the *Optimal Delivery Experiment*, salbutamol delivery is expressed as both percentage (median and range) anticipated total aerosolised salbutamol per series of actuations and micrograms salbutamol per actuation (mean and SD). The fine (<5µm) particle fraction is expressed as a percentage (mean and SD) per actuation and the mean aerodynamic particle size is expressed in µm (mean and SD) per actuation.

Results

Optimal delivery measurement

Data relating to percentage of the anticipated total aerosolised salbutamol (assuming 100µg per actuation) deposited within the spacer and the different stages of the NGI are summarised in Figure 2. The greatest deposition of aerosolised salbutamol was within stage 4 (23% [8-33]) of the NGI, the spacer (21% [8-32]) and stage 3 (17% [6-31]) of the NGI, followed by stages 5 (6% [3-11]) and 2 (6% [2-10]).

The mean (± SD) measured output (per single 100µg actuation) from the MDI was 75±16µg, with a mean calculated aerosol delivery to the NGI of 53±18µg, of which, 48±16µg was within the "fine particle" (<5µm) range, equating to a fine particle fraction of 89.6±2.4%. The mass median aerodynamic diameter (MMAD) of the aerosol, calculated over 16 repetitions (8 from

177 *Experiments 2 and 3*, respectively), was 2.52±0.29μm (namely 50% of the total sample mass
178 was present in particles with aerodynamic diameters <2.5μm, and 50% was present in particles
179 having an aerodynamic diameter >2.52μm, with a geometric standard deviation of
180 1.66±0.16μm.
181
182 *Experiment 1: Effect of shaking the MDI prior to each sequential actuation*
183 There was no significant difference between shaking the MDI at the beginning of 10 actuations
184 and shaking the MDI prior to each of the 10 actuations with regard to percentage of total
185 salbutamol delivered to the NGI (43% [20-66] versus 41% [17-60], respectively) or percentage
186 of total salbutamol delivered to each stage of the NGI (Figure 3).
187
188 *Experiment 2: Effect of angulation of the MDI within the spacer*
189 Compared with a horizontal orientation of MDI output nozzle, there was no significant effect
190 of the other MDI angulations (10° and 20° upward deviation) on percent salbutamol delivered
191 to the spacer, the NGI or the NGI and spacer combined. There was a statistically significant,
192 yet small effect of MDI angulation on the percent salbutamol delivered to stage 8 (P=0.035) of
193 the NGI, whereby the 10° angulation resulted in significantly (P=0.005) less salbutamol
194 delivery than the horizontal orientation (0.4% [0-0.7] vs 0.8% [0.4-1.4]; P=0.005); otherwise
195 there was no significant effect of MDI angulation on salbutamol delivery to any of the NGI
196 stages (Figure 4).
197 When considering only the median drug delivery calculated from the 3 experimental
198 conditions, increasing the MDI angle from a horizontal orientation to a 20° upward deviation
199 resulted in a 15% reduction in total output to the NGI and a 24% increase in retention within
200 the spacer.

Experiment 3: Effect of multiple rapid MDI actuations on salbutamol delivery

Compared with 8 single actuations, there was no significant effect of multiple rapid actuations (4x2 or 2x4) on percent salbutamol delivered to the spacer, the NGI or the NGI and spacer combined. There was a statistically significant, yet small effect of multiple rapid actuations on the percent salbutamol delivered to stages 1 (P=0.007), 2 (P=0.032) and 8 (P=0.032) of the NGI (Figure 5). Four x 2 rapid actuations resulted in significantly less salbutamol delivery to stages 1 (0.7% [0-1.1] vs 1.1% [0.8-1.5]; P=0.021) and 2 (4.9% [2.4-5.7] vs 6.8% [4.4-8.3]; P=0.01) than 8 x single actuations. Two x 4 rapid actuations resulted in significantly less salbutamol delivery to stages 1 (0.1% [0-1.1] vs 1.1% [0.8-1.5]; P=0.007) and 8 (0% [0-1.0] vs 0.5% [0-1.0]; P=0.025) than 8 x single actuations and to stage 8 than 4 x 2 rapid actuations (0% [0-1.0] vs 0.3% [0-1.0]; P=0.021).

When considering only the median drug delivery calculated from the 3 experimental conditions, 4 sets of double rapid actuations resulted in a 15% reduction in total MDI output, 16% reduction in drug delivery to the NGI and 14% reduction to stages 3 and 4 of the NGI. In comparison, 2 sets of quadruple rapid actuations resulted in a 21% reduction in total MDI output, 24% reduction in drug delivery to the NGI and 21% reduction to stages 3 and 4 of the NGI.

Discussion

Despite the increasing popularity of inhalation therapy in the horse, the results of this study highlighted a variety of important considerations with this mode of drug delivery. Importantly, only half of the anticipated MDI output was detected within the NGI. Although a significant proportion of the deficit could be explained by drug retention within the spacer, there remained a proportion which could not be accounted for following sampling from all NGI stages

(including the throat). Therefore, either the MDI did not always achieve a 100µg output during each actuation or drug was deposited within other components of the system which were not subsequently sampled or there was a failure to optimally solubilise all precipitated drug within each NGI component. It is unlikely that significant losses occurred within the tubing between the spacer and NGI. In contrast, significant drug losses may have occurred around the exit nozzle of the MDI because a white residue was often visible at this site during cleaning of the MDI prior to each experiment. Importantly, losses could not be attributed to drug depletion within the MDI as the number of actuations per MDI device were recorded and the MDI replaced well in advance of the calculated drug depletion threshold. This is an important consideration during therapeutic use of such devices as the drug will often become depleted prior to depletion of the propellant (Rubin & Durotoye, 2004).

Despite significant losses within the spacer, the drug delivered to the NGI had a consistently high small particle fraction, with almost 90% of particles being less than 5µm. Furthermore, the calculated MMAD of the aerosol consistently approximated 2.5µm, indicating that 50% of the total sample mass was present in particles with aerodynamic diameters less than 2.5µm and 50% was present in particles having an aerodynamic diameter greater than 2.5µm, with a geometric standard deviation (GSD) of 1.66$\pm$0.16µm. This narrow range of particle size distribution is predicted with an MDI device and contrasts with the more heterodispersed distribution associated with other methods of drug aerosolisation (e.g. ultrasonic, and mesh nebulisation). For example, using the same experimental set up, the authors have demonstrated the generation of an aerosol with a MMAD of 1.4µm and a GSD of 3.2µm using an active mesh nebuliser device[i] commonly used in equine practice (*unpublished observations*).

248 Although the MDI-generated particle size distribution was considered to be optimal for drug
249 delivery to the smaller airways, it should be emphasised that such assumptions, as they relate
250 to equine inhalation therapy, are largely based on human patient derived data. With regard to
251 the prediction of the likelihood of an aerosol penetrating each region of the human respiratory
252 tract, The American Conference of Governmental Industrial Hygienists (ACGIH) describes
253 three fractions (inhalable, thoracic, respirable) generally defined by the aerodynamic diameter
254 at which 50% penetration of that fraction occurs (50% cut-point), with the 50% cut-point for
255 the respirable fraction generally assumed to be 4μm. The likelihood of significant differences
256 between the size-dependent penetration of particles into the equine lung and the human lung
257 has been proposed (Ivester et al., 2014). Although the obligate nasal breathing strategy of
258 horses may predominantly influence the deposition of larger particles, other differences may
259 bring into question the appropriateness of applying human derived data to the horse in relation
260 to the deposition of smaller particles, such as those generated by a MDI. These include the
261 considerably greater (10 to 12-fold) resting tidal volume in the horse and its role in determining
262 linear flow rates within the respiratory tract, with a resultant effect on particle impaction
263 (Ivester et al., 2014). However, in the absence of experimental data to define equine-specific
264 particle fractions or detailed anatomic descriptions of airway dimensions which would permit
265 the construction of predictive models of particle penetration, it is generally assumed that
266 particles less than 4-5μm are likely to reach the lower airways in the horse (Hoffman, 1997;
267 Lavoie, 2001).
268
269 An airflow of 60L/min was used as it more closely approximated the minute volume of an adult
270 horse. The calculation of the particle characteristics (e.g. MMAD and fine particle fraction)
271 was reliant on a constant flow rate through the system and the flow rate applied determines the
272 region of particle deposition within the NGI. However, this differs markedly from the

fluctuating inspiratory flow rates associated with tidal breathing at rest which can typically reach peaks of 120-240L/min. It is likely that the application of a variable flow rate would have had some influence on the degree of drug delivery to the NGI, although the nature of this influence is difficult to predict. Peaks in fluctuating airflow may promote particle impaction at the NGI throat, thus reducing delivery; alternatively, periods of zero flow may facilitate aerosol suspension within the spacer, thus increasing delivery (Duvivier et al., 1997). In human respiratory medicine, the generation of a slow inspiratory flow rate immediately following actuation is recommended to maximise particle delivery to the peripheral airways (Everard et al., 1995; Wildhaber et al., 1996).

Even under optimal delivery conditions, this study revealed a significant degree of variation both in drug retention in the spacer and drug delivery to all stages of the NGI. This variation could not be attributed to repeated use of the MDI as no association was detected between drug output during each series of actuations and the total number of previous actuations of the device (*data not shown*). Although prior knowledge of the predicted losses prior to aerosol delivery to the patient (e.g. within the spacer) will permit some degree of compensation (i.e. delivery of a larger dose), it is not possible to compensate for the unknown delivery achieved with each actuation or series of actuations. The clinical significance of this variation is greatest in relation to the sites of greatest particle deposition; namely within the spacer (CoV - 32%) and stages 3 and 4 (CoV – 49 and 41%, respectively). This equated to a 5-fold difference between the lowest and highest deposition in stages 3 and 4 out of the 16 repetitions performed in the *Optimal Delivery Experiment*. Such variation in delivery has previously been reported by Votion et al., (1997) in relation to both ultrasonic nebulisation and jet aerosol delivery and by Janssens et al., (1999) in relation to MDI delivery via a spacer device in asthmatic children, whereby coefficient of variance values ranging from 23 to 37% were reported, depending on the spacers

used. Such variation will inevitably render any efforts to make accurate dosing recommendations problematic; consequently, recommended doses should be used only as guidelines and the drug should ultimately be administered "to effect" Lavoie, 2001).

All the data used for the *optimal delivery experiment* were derived from experiments 2 and 3, whereby a constant horizontal orientation of the MDI nozzle was maintained within the spacer. Therefore the variation in both aerosol delivery to the NGI and retention within the spacer could not be attributed to the occasional actuation in a suboptimal direction resulting in the high velocity propulsion of drug directly onto the inner surface of the spacer. Furthermore, the coefficient of variance of drug delivered to the NGI in experiment 1, whereby the MDI device was not secured in position, was no greater than that derived from the data included in the *Optimal Delivery Experiment*. Indeed, the results of experiment 2 confirmed that a 20° deviation from the optimal direction of actuation failed to significantly alter the percent salbutamol delivered to the spacer, the NGI or the NGI and spacer combined. Despite this lack of statistical significance, which may partly be attributable to the wide variation in drug delivery between each series of actuations, there was an obvious trend towards a lower drug delivery to the NGI and a greater drug retention within the spacer with increasing angulation of the MDI device.

Owner compliance with respect to the correct use of the MDI device constitutes a major factor in the likelihood of success of inhalation therapy in the horse. Consequently, instructions are regularly provided by the attending clinician, highlighting the "dos" and "don'ts" of MDI and spacer use which are largely based on recommendations applied within the medical profession. These generally involve factors such as shaking the MDI prior to each actuation, exhaling fully prior to actuation, holding the MDI vertically, coinciding actuation with inspiration, adopting a slow inspiratory effort, initiating only a single actuation per breath, and subsequent breath holding for a minimum of 5s (Resnick et al., 1996). However, despite the relative simplicity of

these steps, several studies have revealed poor knowledge of correct MDI use protocol, particularly amongst medical professionals (Jones et al., 1995; Resnick et al., 1996; Stelmach et al., 2007). Furthermore, certain studies have identified particular recommendations to be inappropriate and potentially detrimental with regard to their potential influence on user compliance (Everard et al., 1995). Such recommendations are likely to have greater influences on compliance when they significantly increase the time required for drug administration; particularly in equine inhalation therapy when multiple actuations are required.

Everard et al., (1995) clearly demonstrated the importance of MDI shaking prior to drug administration, likely reflecting the importance of mixing the active drug and the propellant within the MDI device. However, the current study failed to demonstrate any significant benefit of shaking the MDI device before each actuation in a series of sequential actuations, in relation to both total and respirable particle delivery to the NGI. This finding likely reflects an insufficient time period (5s) between each actuation to permit separation of the salbutamol and the propellant. Everard et al., (1995) also demonstrated a reduction in both total and respirable particle generation with multiple actuations in rapid succession. Although the current study failed to identify a statistically significant effect of rapid double and quadruple actuations on drug delivery to the spacer, NGI or spacer and NGI combined, there was a trend for increased rapid sequential actuations to reduce drug delivery. However, in light of the small magnitude of the reduction, this could readily be compensated for by a small increase the number of actuations; for example, an extra actuation for every 4 rapid consecutive actuations.

In conclusion, this study demonstrates the difficulties in predicting the magnitude of drug delivery to the peripheral airways using an MDI and equine spacer device. Therefore, when selecting the most appropriate route of drug administration, this shortcoming must be considered and weighed up against the advantages of this therapeutic approach, including the reduced risk of systemic adverse drug effects and relatively lower drug costs. Furthermore, the

apparent lack of requirement to shake the MDI prior to each actuation and the limited effect of multiple rapid actuations can significantly reduce the time required to administer the treatment and therefore have the potential to improve owner compliance with regard to MDI use in the horse. Finally, it is important to appreciate that any conclusions derived from this study can only be applied clinically to the use of salbutamol. It remains unknown whether similar results would be obtained with other drug aerosols generated by an MDI device (e.g. corticosteroids) as differences in viscosity, density and surface tension have the potential to influence both the particle size distribution of the aerosol generated as well as the rapidity with which the drug and propellant separate between actuations.

Manufacturers

[a] Next Generation Impactor, Copley Scientific, Colwick Quays Business Park, Private Road No. 2, Colwick,Nottingham, NG4 2JY, United Kingdom

[b] Ventolin® Evohaler® 100 micrograms, Glaxo Wellcome UK Limited, Stockley Park, West Uxbridge Middlesex UB11 1BT

[c] Equine Haler®, Jørgen Kruuse A/S, Havretoften 4 DK-5550 Langeskov Denmark

[d] Copley High Capacity Pump, Copley Scientific, Colwick Quays Business Park, Private Road No. 2, Colwick,Nottingham, NG4 2JY, United Kingdom

[e] S5013 Sigma-Aldrich Company Ltd. Dorset, England

[f] Corning® 3675 96 well plates, UV-transparent , Sigma-Aldrich Company Ltd. Dorset, England

[g] Synergy HT Biotek, BioTek Instruments Inc 2005, Papermakers House, Rivenhall Road, Swindon SN5 7BD, United Kingdom

[h] Copley Inhaler Testing Data Analysis software (CITDAS), Copley Scientific, Colwick Quays Business Park, Private Road No. 2, Colwick,Nottingham, NG4 2JY, United Kingdom

[i] Flexineb, Nortev, Unit 18, Claregalway Corporate Park, Galway H91 KFX3, Ireland

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443

Figures

Figure 1 (a-c): Experimental setup. (a) The spacer (S) was fixed to the throat (black arrow) of the NGI in a horizontal position and the MDI was inserted into the spacer adjacent to the inspiratory valves (red arrow). (b) The NGI was comprised of a throat (not shown) and a series of eight stages through which air (containing the generated aerosol) flowed at a constant flow rate via pore sizes of sequentially decreasing diameter and consisting of eight particle collection plates (labelled 1-8). (c) For *Experiment 2*, the orientation of the MDI nozzle relative to the horizontal position was determined by its attachment to a combination square angle finder which was fixed to the bench.

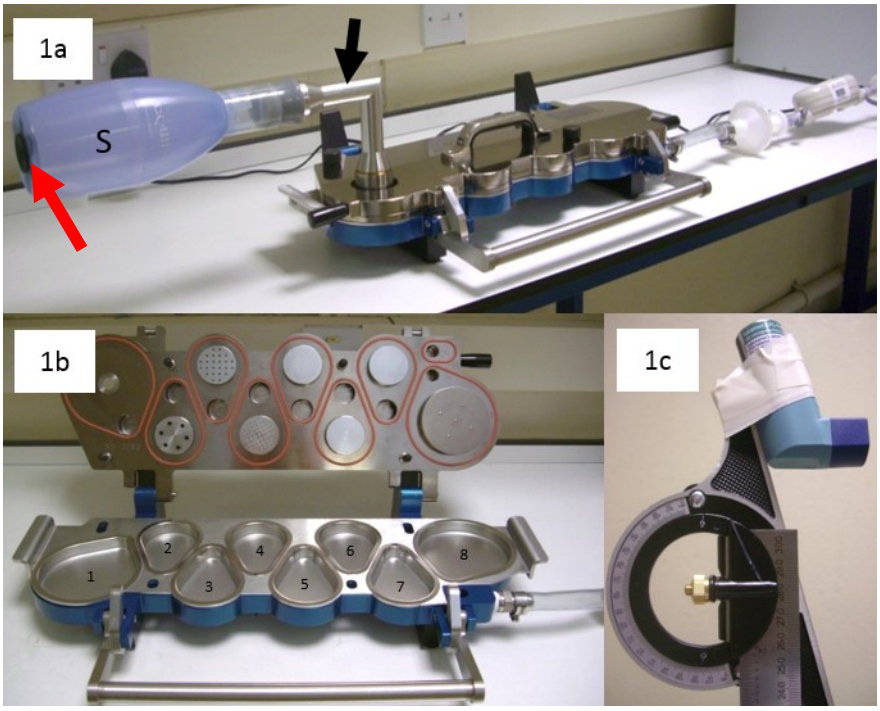

Figure 2: Box and whiskers plot depicting the percentage of anticipated total aerosolised salbutamol (assuming 100µg per actuation) deposited within the spacer and the different stages of the NGI (throat and stages 1 – 8) under perceived optimal conditions. Data were derived from Experiments 2 and 3 when the MDI nozzle was orientated in a horizontal position with a 5 s delay between actuations. Median (horizontal line), interquartile range (box limits) and range (whisker limits) derived from 2 x 8 repeats of either 10 *(Experiment 2)* or 8 *(Experiment 3)* actuations.

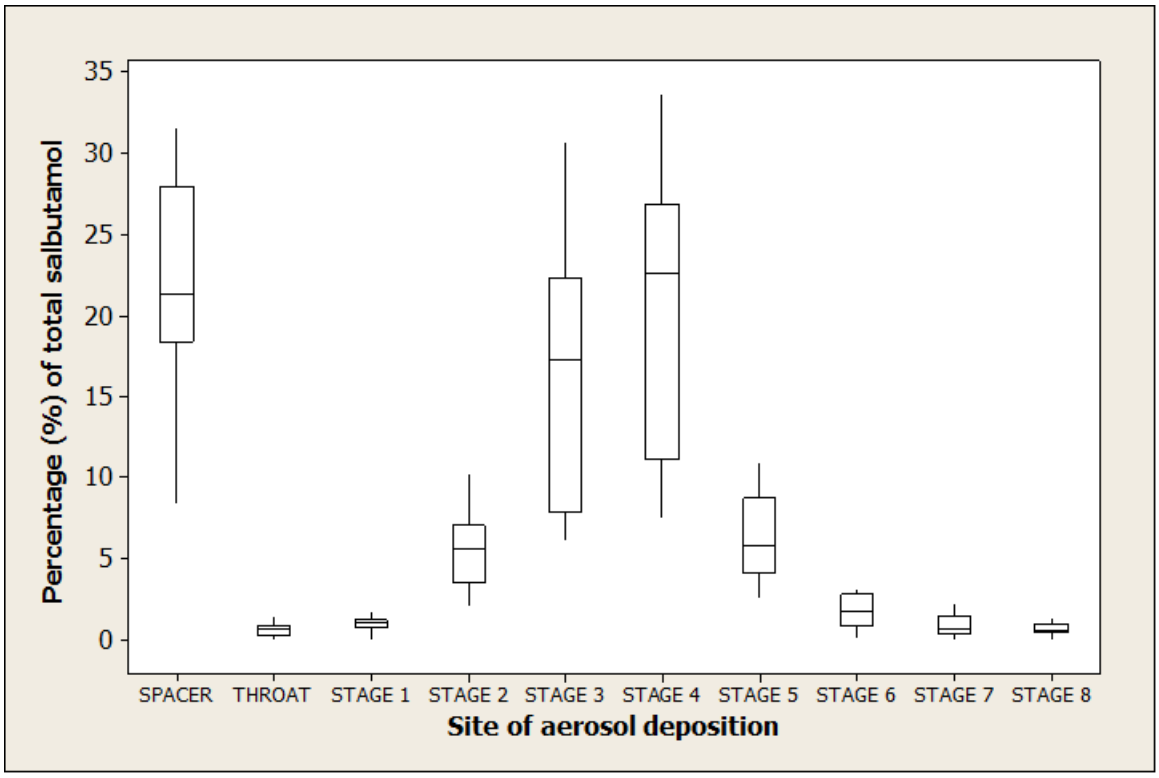

Figure 3: Box and whiskers plot depicting the percentage of anticipated total aerosolised salbutamol (assuming 100µg per actuation) deposited within the different stages of the NGI (throat and stages 1 – 8) when the MDI was either shaken for 30s prior to the first actuation and then actuated at 5 s intervals without further shaking (solid boxes) or shaken for 30s prior to each actuation (open boxes). Median (horizontal line), interquartile range (box limits) and range (whisker limits) derived from 2 x 8 repeats of 10 actuations. Asterisk = outlier.

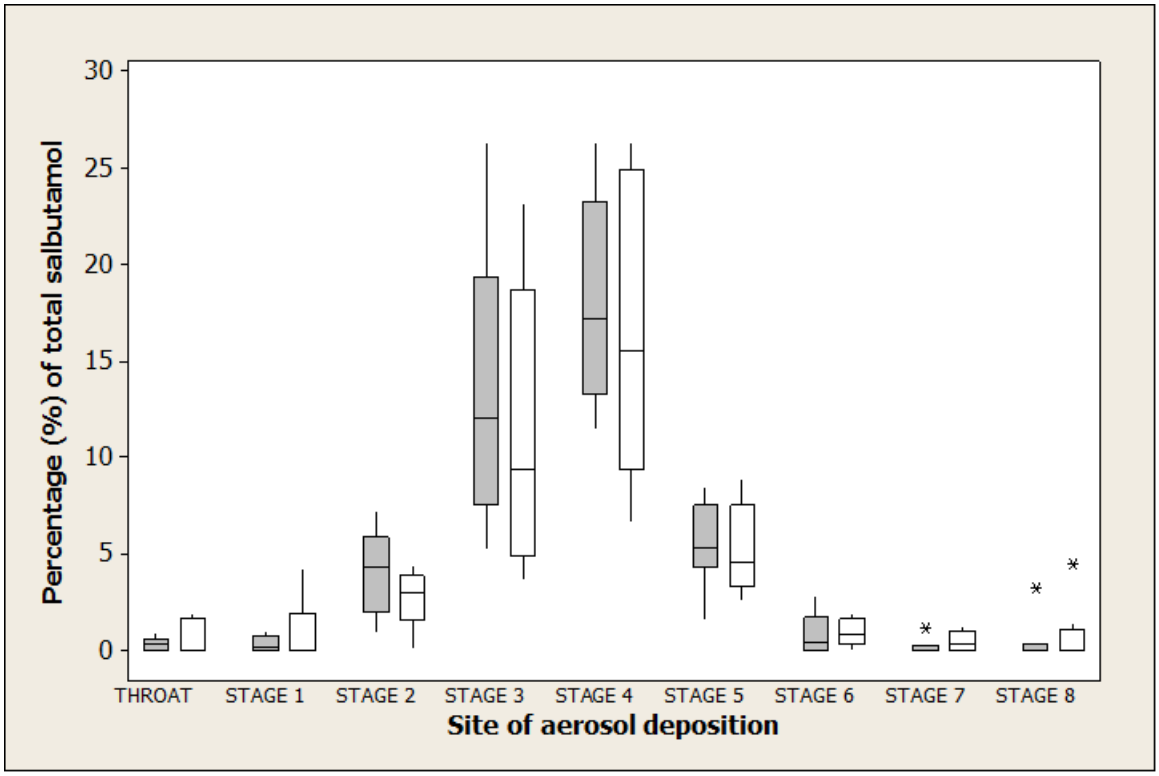

Figure 4: Box and whiskers plot depicting the percentage of anticipated total aerosolised salbutamol (assuming 100µg per actuation) deposited within the spacer and the different stages of the NGI (throat and stages 1 – 8) following actuation of the MDI device when the output nozzle was horizontal (open boxes) or deviated 10° (hatched boxes) or 20° (solid boxes) above the horizontal. Median (horizontal line), interquartile range (box limits) and range (whisker limits) derived from 3 x 8 repeats of 10 actuations. Asterisk = outlier; horizontal bar – limits depict significantly different data sets (P<0.05).

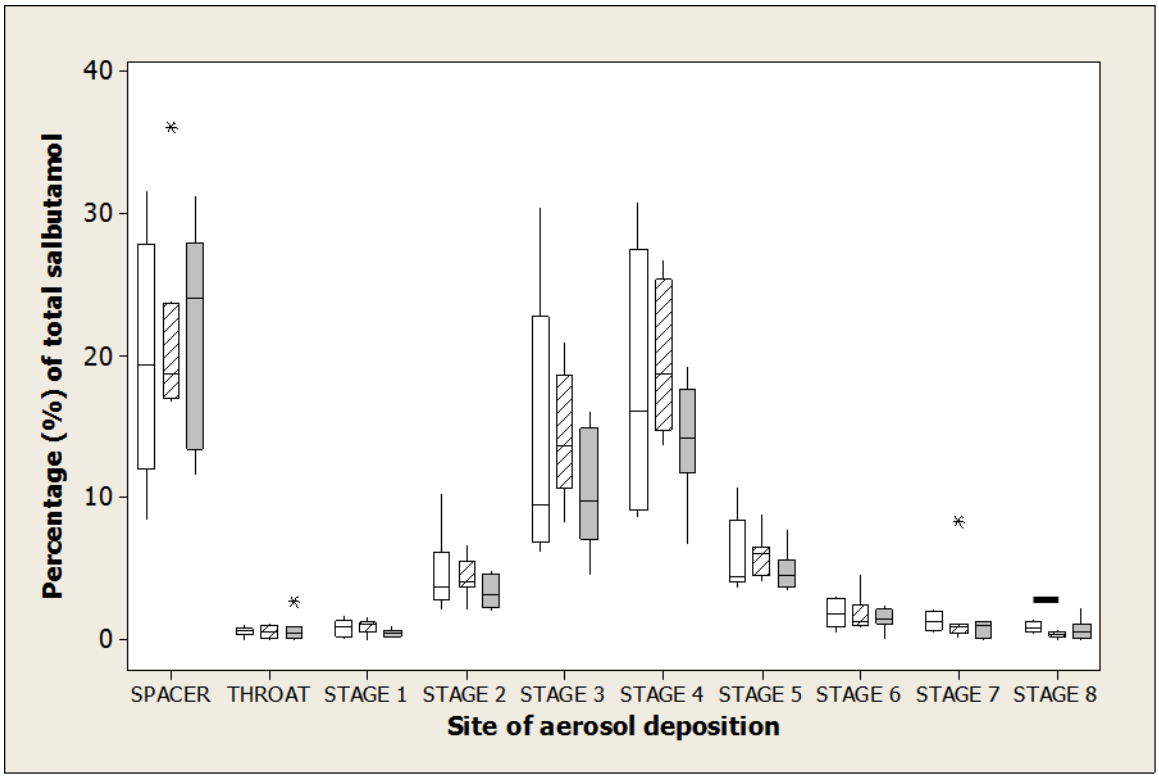

Figure 5: Box and whiskers plot depicting the percentage of anticipated total aerosolised salbutamol (assuming 100µg per actuation) deposited within the spacer and the different stages of the NGI (throat and stages 1 – 8) following 8 actuations, either delivered individually at 5s intervals (open boxes), as 4 x double actuations in rapid succession (hatched boxes) or as 2 x quadruple actuations in rapid succession (solid boxes). Median (horizontal line), interquartile range (box limits) and range (whisker limits) derived from 3 x 8 repeats of 8 actuations. Asterisk = outlier; horizontal bar – limits depict significantly different data sets ($P<0.05$).

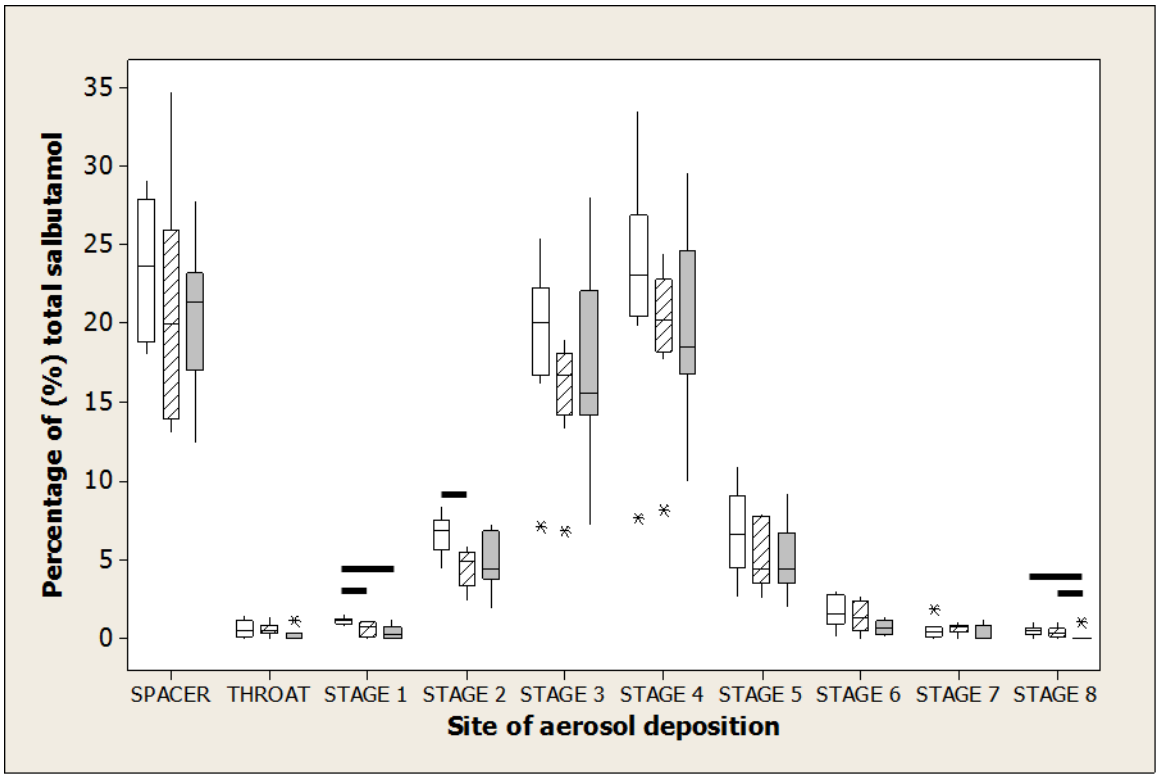

Table 1: Summary of experimental designs. Shaded cell indicates the comparisons made for each experiment. Bold text indicates the data used to measure aerosol characteristics in the Optimal Delivery Experiment.

	MDI shake	**MDI angulation**	**Rapidity of successive actuations**
Experiment 1	single shake prior to series of 10 actuations *versus* shake prior to each actuation	angle of actuation not standardised	10 x single actuations
Experiment 2	single shake prior to series of 10 actuations	**horizontal** *versus* 10° upward angulation *versus* 20° upward angulation	10 x single actuations
Experiment 3	single shake prior to series of 8 actuations	horizontal	**8 x single actuations** *versus* 4 x double rapid actuations *versus* 2 x quadruple rapid actuations
Optimal Delivery Experiment	single shake prior to series of 8 or 10 actuations	horizontal	8 or 10 x single actuations